Array comparative genomic hybridization with cyanin cis-platinum-labeled DNAs

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Fluorescent cis-platinum compounds that react with the N7 atom of guanine are useful for labeling nucleic acids in fluorescence hybridization applications. Here we report that cyanin (CyN) cis-platinum labeling of DNA samples for array comparative genomic hybridizations (arrayCGH) can be achieved reproducibly and reliably. We demonstrate that degrees of labeling of approximately 1% of all nucleotides in test and reference DNA samples with CyN3- and CyN5-cis-platinum produces arrayCGH signal-to-background ratios ranging from 30 to 40. The arrayCGH results achieved during analyses of mouse and human tumor samples were comparable to those achieved using enzymatic labeling. Thus, we conclude that Cy-cis-platinum labeling is an alternative to enzymatic labeling for arrayCGH.

INTRODUCTION

Fluorescent labeling of nucleic acid targets is a crucial element of microarray hybridizations for determining copy numbers of genome segments and gene transcripts (1–3). In most applications, enzymatic incorporation of fluorescent precursors such as cyanin3 (CyN3)- and CyN5-dNTPs is employed to generate differentially labeled nucleic acids for use in bicolor hybridization formats. Particularly in the RNA expression profiling field, the realization that different fluorescent dNTPs may have different incorporation efficiencies has led to the use of allylamine-dUTP for enzymatic generation of amino-modified target nucleic acids such that test and reference nucleic acids are amino-modified to the same extent (4). In a subsequent chemical step, these amino-modified polynucleotides are reacted with amino-reactive members of a fluorescent dye family such as the cyanins, notably their N-hydroxysuccinimide esters. In all, this permits generation of differentially labeled test and reference targets with identical degrees of labeling (DOLs) and consequently identical hybridization and duplex stability properties [i.e., melting temperatures (Tm/s)]. In self-self hybridizations, any difference in fluorescent hybridization signals of the two channels would then be caused by differences in dye quantum yield and spectral characteristics of the light source and the detection device. In test reference hybridizations, this leads to more reliable copy number difference determinations and obviates color swap experiments for analyzing dye incorporation biases.

Fluorescent cis-platinum compounds that react monofunctionally with electronegative moieties in nucleic acids, notably N7 of guanine residues, enable one-step chemical labeling of nucleic acids (5), and next to fluorescence in situ hybridization (FISH) (6,7) and chromosomal comparative genomic hybridizations (CGHs) (8), monofunctional labeled cis-platinum compounds have found application in direct mRNA and first strand cDNA labeling for gene expression profiling by microarray hybridizations with no dye bias (9,10).

Recently we have analyzed CyN3- and CyN5-cis-platinum DNA adducts free in solution in terms of thermal duplex stability, fluorescence yields, and kinetics of formation (11). It was determined that effects of CyN-cis-platinum modifications on Tm were quite moderate (0.5°C per percent modification), that self-quenching occurs at DOLs ≥2%–3%, and that at 85°C adduct formation obeys (pseudo) first order kinetics with half-lives of a few minutes. This implies that end point labeling can be achieved in 20–30 min, thus allowing reproducible labeling at

Figure 1. Cyanin3 (CyN3)- and CyN5-cis-platinum DNA labeling properties. Male (CyN5) and female (CyN3) human DNAs were end point-labeled at predetermined degrees of labelings (DOLs) by adding 1/DOL mol CyN-cis-platinum per mol DNA (nucleotide) and a 30-min incubation at 85°C.
predetermined DOLs. These “in solution” results were confirmed in solid phase hybridizations using cis-platinum-labeled oligonucleotides and microarrays with spotted complementary oligonucleotides (11).

In this study, we have used chemical cis-platinum labeling of genomic DNA

Figure 2. Effect of the degree of labeling (DOL) on average spot (S) and background (B) intensities. A selection of the DNAs of Figure 1 was hybridized to low complexity human arrays. Squares, cyanin3 (CyN3); triangles, CyN5; open symbols, average spot intensity; gray symbols, average background intensity; black symbols, S/B. a.u., arbitrary units.

Figure 3. Effect of averaged cyanin3 (CyN3) and CyN5 degrees of labeling (DOL) on averaged CyN3 and CyN5 signal-to-background ratio, standard deviation (sd) of the average log2(ratio) of the autosomal clones, and the X clone amplitude. Squares, CyN3/CyN5 averaged S/B; circles, X amplitude; triangles, sd of the average of log2(ratio autosomal); black symbols, data of the DOL series of Figure 2 with the DNAs hybridized to low-complexity arrays; gray symbols, data from three independent CyN-cis-platinum labeling experiments and hybridizations to complex human arrays; white symbols, data from three independent enzymatic labeling experiments and hybridizations to complex human arrays.
for complex genomic DNA microarray hybridizations and compare this procedure with enzymatic labeling.

**MATERIALS AND METHODS**

We used small and large human genomic arrays prepared essentially according to Fiegler et al. (12) and employed the Leiden Genome Technology Center (Leiden, The Netherlands) facilities for clone manipulation, amplification, and array printing (http://www.lgtc.nl). The small, low complexity human array consisted of 110 autosomal subtelomeric and X chromosomal bacterial artificial chromosome (BAC)/P1 artificial chromosome (PAC) DNAs spotted in triplicate and the large, high complexity microarray of about 3100 BAC/PACs essentially as described by Fiegler et al. (12) spotted in triplicate. Details of the clones selected can be obtained from the Ensembl database accessed in the CytoView pages (http://www.ensembl.org/Homo_sapiens/cytoview). Normal male and female DNAs were from Promega (Leiden, The Netherlands). The human cutaneous lymphoma DNA was obtained from the Department of Dermatology of the Leiden University Medical Center (Courtesy Dr. Maarten Vermeer). Mouse genomic microarrays are described in Hodgson et al. (13), as is the mouse islet tumor DNA used here.

Enzymatic random-prime labeling was performed according to Fiegler et al. (12) and leads in our hands to DOLs of approximately 1.5% for CyN3 and CyN5. The CyN-cis-platinum were generous gifts of Kreatech Biotechnology (Amsterdam, The Netherlands).

Before cis-platinum labeling, a cold random prime reaction was performed with nonmodified dNTPs and 500 ng DNA. Following purification with a QiAquick® PCR purification kit (Qiagen, Valencia, CA, USA) using 10 mM Tris-HCl, pH 7.4, as the elution buffer, the DNA concentration was measured using a UV spectrophotometer (2100 pro UV/Vis; Amersham plc, Little Chalfont, Buckinghamshire, UK).

**Figure 4. Ratio profile comparisons of enzymatic and cis-platinum labeling methods.** Ratio profiles were obtained with complex human and mouse genomic bacterial artificial chromosome (BAC)/P1 artificial chromosome (PAC) arrays. Left panels, enzymatic labeling. Right panels, cis-platinum labeling. (A and B) Normal female to male human DNA hybridization. (C and D) Mouse tumor to normal mouse DNA hybridization. (E and F) Human cutaneous lymphoma and normal human DNA.
equipped with a microcell that accommodates 5–10 μL sample volumes. The amount of cis-platinum needed for the desired DOL was calculated using molecular weights for CyN3- and CyN5-cis-platinum of 1019 and 1045 Da, respectively. cis-Platinum end point labeling reactions were performed at approximately 0.1 μg DNA/μL for 30 min at 85°C using a PCR apparatus with a heated lid. Following Sephadex® G-50 Spin column purification (Amersham plc), UV-Vis spectra were recorded, and DOLs calculated using correction factors as described (11).

CyN3- and CyN5-cis-platinum-labeled DNAs (4 μg each for small human and the mouse arrays and 10 μg each for large human arrays) were then combined, ethanol precipitated in the presence of 10× excess Cot-1 DNA (Roche Diagnostics, Mannheim, Germany), and dissolved in 60 μL of 50% formamide, 2× standard saline citrate (SSC), 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), 10 μg/μL yeast tRNA. After 10 min denaturation at 75°C, the DNA was preannealed at 37°C for 30–60 min. Microarrays were prehybridized for 60 min at 37°C with denatured 40 μg/μL herring sperm DNA and 1 μg/μL Cot-1 DNA in hybridization buffer. Prehybridization was performed in a moist chamber, and prehybridization areas were defined by Frame-Seal Chambers (MJ Research, Waltham, MA, USA). Agitation of the prehybridization solution was achieved by a rocking device. Hybridization of the labeled DNAs to microarrays was for 48 h at 37°C. Washes were with 50% formamide, 2× SSC, pH 7.0, for 15 min at 48°C; 2× SSC, 0.1% SDS for 30 min at 48°C; 0.1 M sodium phosphate buffer, pH 8.0, with 0.1% Igepal (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 15 min at room temperature; and finally with water. The arrays were then air-dried.

Scanning was with an Axon 4100A scanner (Axon, Union City, CA, USA) equipped with emission filters for CyN3 and CyN5 (bandwidths 550–600 nm, resp. 655–695 nm). Throughout this study, the photomultiplier gains were set at 700 V for the CyN3 channel and at 800 V for the CyN5 channel, and scanning resolution was always 10 μm. Pixel intensity data, averaged over five scans, were used in GenePix® version 4.0 (Axon) for obtaining median intensity data of the spots and their local backgrounds. For the human arrays, Excel® macros were used for data analysis and generating graphs of log2(ratio) plots. For mouse arrays, the Spot software described by Jain et al. (14) was used.

RESULTS AND DISCUSSION

Normal female and male human DNAs were labeled with CyN3- and CyN5-cis-platinum at different predetermined DOLs. From the linear relationship between the CyN3- and
CyN5-cis-platinum DOLs shown in Figure 1, it is clear that with the end point labeling format, CyN3 and CyN5 DOLs can be obtained that differ only a few tens of percentages when labeling with identical molar ratios of CyN-cis-platinum over DNA.

Following co-hybridization of DNAs with near identical DOLs to the low complexity human arrays, the effect of the DOL on average median spot and background intensity and the signal-to-background (S/B) ratio was analyzed (Figure 2). Maximum S/B is obtained at a DOL of approximately 1% for both cis-platinum. Also, the actual maximum S/B values were quite similar and high with values up to 40. At higher DOLs, S/Bs decreased due to quenching of fluorescence (11) and slight increases in background intensities. With the mouse arrays, similar results were obtained.

Figure 3 gives for the DOLs tested the standard deviations of the log2(ratio_autosome) as a measure of the precision with which copy number differences can be detected and the average log2 ratio of the X chromosome clones (X clone amplitude) as a measure of the capacity to quantify relative copy number. Overall we found that at S/B > 10, these general performance parameters stabilized to values of 0.01 and 0.6 for both labeling approaches.

On the basis of these results, we used CyN-cis-platinum DOLs of approximately 1% for comparison with enzymatic labeling protocols in arrayCGH with complex arrays. Three independent labeling reactions were executed for the enzymatic and chemical labeling, and complex human BAC arrays were used for evaluation. The results are also shown in Figure 3, while in Figures 4, A and B, representative arrayCGH ratio profiles of chemical and enzymatic labeling are presented of these normal human male and mouse female to normal male hybrids. Again the performance parameters of the two labeling methods proved to be quite similar (Figure 3), and on basis of these results, we concluded that the cis-platinum labeling methodology performs at least as well as the enzymatic one. As a final proof of this, we analyzed several tumor DNAs of human and mouse origin. As can be seen from the two examples shown in Figure 4, for a mouse tumor-derived cell line DNA (panels C and D) and human cutaneous lymphoma DNA (panels E and F), the ratio profiles obtained with the two labeling methods are nearly identical. Several of these experiments were color-swapped with identical results (data not shown). In general, in routine experiments with chemical and enzymatic labeling, we find that slight variations in results are attributable to other factors than the labeling protocol per se.

In summary, we have shown that chemical cis-platinum end point labeling allows for controlled and reproducible labeling of genomic DNAs, that the S/B ratios in arrayCGH with CyN3- and CyN5-cis-platinum-labeled DNAs are maximal at approximately 1% DOL, and that with two types of genomic arrays (i.e., mouse and human arrays produced by two different centers), such CyN-cis-platinum-labeled DNAs perform equivalently to enzymatically CyN-labeled DNAs in arrayCGH. Hence, CyN-cis-platinum labeling of genomic DNA for arrayCGH may be considered for incorporation in arrayCGH protocols.

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REFERENCES


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